A new ent-kaurane diterpenoid from *Isodon nervosus*

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A new *ent*-kaurane diterpenoid, 6β,7β,13α-trihydroxy-1α-acetoxy-7α,20-epoxy-*ent*-kaur-16-en-15-one (**1**) and five known compounds, epinodosinol (**2**), isodocarpin (**3**), nodosin (**4**), enmedol (**5**), nervosanin B (**6**), were isolated from the aerial part of *Isodon nervosus.* The structure of the new compound was determined by spectral methods (1D-,2D-NMR and MS). Four of the compounds were tested for their cytotoxicity towards HL60, SMMC-7721 and Hela cells.

Keywords: labiatae, *Isodon nervosus,* ent-kaurane, diterpenoid, cytotoxicity

Isodon species are widely distributed in China and some 10 species have been used in Traditional Chinese Medicine for the treatment of gastrointestinal disorders and more recently as antitumor agents.[1,2] Our previous phytochemical studies on plants of this genus have led to the isolation of more than 10 new *ent*-kaurane diterpenoids,[3–5] some of which showed potential cytotoxic activity *in vitro*. Further investigation of the aerial part of *Isodon nervosus* led to the isolation of a new ent-kaurane diterpenoid, 6β,7β,13α-trihydroxy-1α-acetoxy-7α,20-epoxy-*ent*-kaur-16-en-15-one (**1**) and five known compounds, epinodosinol (**2**), isodocarpin **(3)**, nodosin (**4**), enmedol (**5**) and nervosanin B (**6**) were isolated. In addition, four of the compounds (**1**–**4**) were tested for their cytotoxicity towards human leukaemia cell (HL60), human hepatoma cell (SMMC-7721) and human cervical carcinoma cell (HeLa). This report describes the structure determination of compound **1** and cytotoxicity of compounds (**1**–**4**).

The leaves of *Isodon nervosus* (Hemsl.) were collected from Tongbai prefecture of Henan province, China, in August 2006. The plant material was identified by Professor Changshan Zhu, Henan Agriculture University, P. R. China. A voucher specimen (No. 200609) has been deposited in the Pharmacy College, Xinxiang Medical University. The dried and powdered leaves of *Isodon nervosus* were extracted with Me_2CO/H_2O (7 : 3 v/v) at room temperature, and the extract subjected to column chromatography over silica gel to give compounds **1**–**6**.

Compound **1** exhibited a molecular formula $C_{22}H_{30}O_7$ based on its HR-ESI-MS at *m/z* 429.1879 $[M + Na]^+$ (Calcd 429.1889). The IR spectrum revealed the presence of carbonyl groups (1714 cm^{-1}) and hydroxyl groups (3400, 3318 and 3268 cm^{-1}). In the ^1H, ^{13}C and DEPT NMR spectra, in addition to the signals of an acetoxy group at δ 169.7 (s), 21.4 (q), 1.91 (3H, s), there were 20 carbon signals which included two tertiary methyls, seven methylenes (including one oxygenated at δ 68.8 (t) and one olefinic carbon at δ 116.7 (t)), four methines (including two oxygenated carbons at δ 71.1 (d), 74.5 (d)), seven quaternary carbons (including one oxygenated carbon at δ 74.1 (s)), one carbonyl carbon at δ 208.5 (s), one olefinic quaternary carbon at δ 156.4 (s)), and one hemiketalic carbon at δ 96.2 (s)). This suggested that **1** was a 7,20-epoxy-*ent*-kaurenoid by comparison with reported ^{13}C NMR spectral data.[6,7] The position of the acetoxy group was confirmed at C-1 (δ 71.1 d) from the long–range correlation of H-1 (δ 5.56 m) with C-21 (δ 169.7 s) in the HMBC spectrum, and three hydroxyl groups were attached to C-6, C-7 and C-13 from the HMBC correlations of H-6 (δ 4.3 br s) with C-5 (δ 60.3 d), C-7 (δ 96.2 s) and C-8 (δ 60.7 s); H-20 (δ 4.23, 4.45 d) with C-7 (δ 96.2 s); H-11(δ 1.17 m), H-14 (δ 2.97, 2.75 d) and H-17 (δ 6.22, 5.89 br s) with C-13 (δ 74.1 s), respectively.

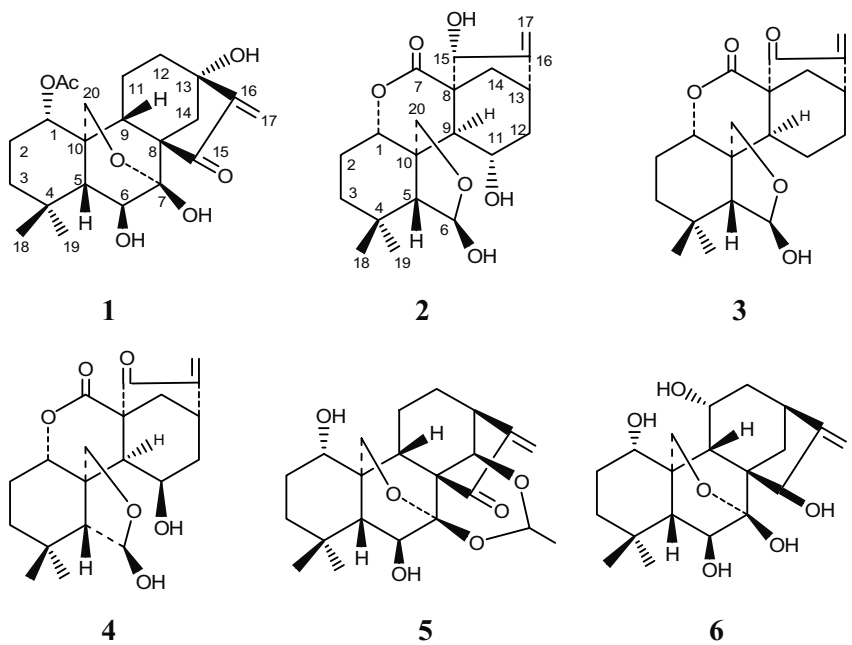

Fig. 1 The structures of compounds **1**–**6**.

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The literature[8–10] showed that the H-5 and H-9 were β-oriented and H-13 was in α-orientation in the 7,20-epoxy-*ent*-kaurane-type diterpenoids. Hence, the 13-OH should be α-orientation in compound **1**. The observed NOESY correlations from H-1 to H-9 and OAc to H-20, established that H-1 had the β-orientation; The observed NOESY correlation from H-6 to H-19 and the absence of a correlation with H-5, showed that the H-6 had the α-orientation (Fig. 2). Therefore, the structure of **1** was determined to be 6β,7β,13α-trihydroxy-1α-acetoxy-7α,20-epoxy-*ent*-kaur-16-en-15-one (Fig. 1).

Compounds **2**–**4** were identified by comparison of their ^{1}H and ^{13}C NMR, MS and IR spectroscopic data with those reported in literatures as epinodosinol (**2**),[11] isodocarpin (**3**),[12] nodosin (**4**),[13] enmedol (**5**),[14] and nervosanin B (**6**).[15]

Using SRB method,[16] the anti-tumour activities of compounds **1**–**4** against HL60, SMMC-7721 and Hela cells were assayed by comparison with the standard Mitomycin. From the anti-tumour activity data (Table 2), it was found that isodocarpin (**3**) and nodosin (**4**) were significantly cytotoxic against the HL60 cells with IC_{50} value of 0.57 μg ml^{-1} and 2.08 μg ml^{-1} respectively.

Experimental

Melting points were determined with a Kofler melting point apparatus and are uncorrected. Optical rotations were measured on a Perkin-Elmer 341 polarimeter. UV spectra were recorded on a Shimadzu UV-2550 instrument. IR spectra were taken on a Nicolet 170 SX FT-IR spectrometer. ^{1}H, ^{13}C and 2D NMR spectra were recorded on a Bruker AM-400 NMR spectrometer with TMS as internal standard. HR-ESI-MS was obtained on a Waters HPLCQ-Tof HR-MS spectrometer. Silica gel (200–300 mesh) was used for column chromatography and silica gel GF_{254} for TLC were made by the Qing-dao Marine Chemical Factory of China.

Extraction and isolation procedures

The air-dried leaves of *Isodon nervosus* (14 kg) were pulverised and extracted three times with Me_2CO/H_2O (7 : 3 v/v) at room temperature for 3 days and filtered. The filtrate was concentrated and partitioned with EtOAc. The EtOAc layer was evaporated under reduced pressure to obtain a residue (300 g), which was absorbed on 600 g silica gel and subjected to silica gel column (10 × 120 cm, 3000g, 200–300 mesh) eluted with a gradient of $CHCl_3$–CH_3OH (1 : 0, 30 : 1, 20 : 1, 10 : 1, 5 : 1; 3 : 1, 0 : 1) to give seven fractions according to their TLC analysis. Compound **1** (31 mg)**, 3** (14 mg)**, 4** (225 mg) and **5** (12 mg) were obtained from the fraction 3 ($CHCl_3$–CH_3OH 20 : 1) by repeated silica gel column with $CHCl_3$–$(Me)_2CO$ or $CHCl_3$–CH_3OH. Compound **2** (40 mg) and **6** (9 mg) were obtained from the fraction 4 ($CHCl_3$–CH_3OH 10 : 1) by repeated silica gel column with $CHCl_3$–$(Me)_2CO$ or $CHCl_3$–CH_3OH.

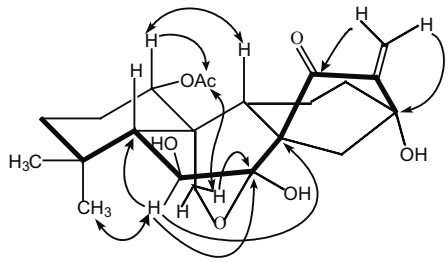

Fig. 2 The key Noesy and HMBC of compound **1.**

6β,7β,13α-Trihydroxy-1α-acetoxy-7α,20-epoxy-ent-kaur-16-en-15-one (**1**): Colourless needles, m.p. 265–266 °C; $[\alpha]_D^{23}$–97.0 (*c* 0.11, CH_3OH); UV λ_{max} (MeOH) 230 (logε, 3.72) nm; IR (KBr) ν_{max}/cm^{-1}: 3400, 3318, 3268, 1714, 1650, 1377, 1265, 1056; HR-ESI-MS Found: 429.1879, Calcd. for $C_{22}H_{30}O_7$ + Na: 429.1889. For ^{1}H and ^{13}C NMR data see Table 1.

Anti-tumour activity assays

The cytotoxicity of compounds **1**–**4** toward HL60, SMMC-7721 and Hela cells were determined in 96-well microtitre plates by the sulforhodamine B method.[16] Briefly, exponentially growing HL60, SMMC-7721 and Hela cells were harvested and seeded in 96-well plates with the final volume 100 μl containing 4 × 10^3 cells per well. After 24 h incubation, cells were treated with various concentrations

Table 1 ^{1}H NMR(400 MHz),^{13}C NMR (100 MHz) and HMBC data of compound **1** (C_5D_5N, δ in ppm)

Position	δ_C	δ_H	HMBC
1	71.1d	5.56 (1H,m)	H-AcO,20
2	31.0 t	1.47, 1.23 (2H,m)	H-3,18,19
3	41.6 t	1.35 (2H,m)	H-5,18,19
4	33.9 s		H-2,5, 18,19
5	60.3 d	1.42 (1H,d,*J* = 7.2 Hz)	H-3,6,18,19,20
6	74.5 d	4.30 (1H,brs)	H-5
7	96.2 s		H-5,6,14,20
8	60.7 s		H-6,9,20
9	53.0 d	1.79 (1H,d, *J* = 4.4 Hz)	H-1,12,14,20
10	37.1 s		H-2,6, 9,20
11	18.8 t	1.17 (2H,m)	H-1
12	47.0 t	2.50, 2.46 (2H,d, *J* = 5.2 Hz)	H-9,14,17
13	74.1 s		H-11,14,17
14	36.8 t	2.97, 2.75 (2H,d,*J* = 11.6,16 Hz)	H-9,12
15	208.5 s		H-9,14,17
16	156.4 s		H-12,14,17
17	116.7 t	6.22, 5.89 (2H,brs)	H-14
18	34.5 q	1.29 (3H,s)	H-3,5,6,19
19	22.7 q	1.07 (3H,s)	H-3,5,6,18
20	68.8 t	4.23, 4.45 (2H,d,*J* = 8.8 Hz)	H-5,9
OAc	169.7 s	1.91 s	H-1
	21.4q		

Table 2 Anti-tumour activity of compounds **1**, **2**, **3** and **4**

Compd	IC_{50} (μg ml^{-1})		
	HL60	SMMC-7721	Hela
1	24.93 ± 4.13	45.18 ± 3.04	89.12 ± 3.97
2	26.44 ± 1.99	39.68 ± 5.64	59.90 ± 1.69
3	0.57 ± 0.12	3.57 ± 0. 26	4.01 ± 0.67
4	2.08 ± 0.34	25.69 ± 1.42	12.67 ± 2.67
Mitomycin	0.56 ± 0.17	1.85 ± 0.53	1.11 ± 0. 64

of compounds for 48 h. The cultures were fixed at 4 °C for 1 h by addition of ice-cold 50% trichloroacetic acid (TCA) to give a final concentration of 10%. Fixed cells were rinsed five times with deionised water and stained for 10 min with 0.4% sulforhodamine B dissolved in 0.1% acetic acid. The wells were washed five times with 0.1% acetic acid and left to dry overnight. The absorbed sulforhodamine B was dissolved in 150 µl unbuffered 1% Tris base [tris(hydroxymethyl) aminomethane] solution in water (pH 10.5). The absorbency of extracted sulforhodamine B at 515 nm was measured on a microplate reader (Bio-Rad). The experiments were carried out in triplicate. Each run entailed 5–6 concentrations of the compounds being tested. The percentage survival rates of cells exposed to the compounds were calculated by assuming the survival rate of untreated cells to be 100%.

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